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Increased Interleukin-6 Production in Mouse Osteoblastic MC3T3-E1 Cells Expressing Activating Mutant of the Stimulatory G Protein

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ABSTRACT

The McCune–Albright syndrome (MAS) is characterized by polyostotic fibrous dysplasia, café-au-lait spots, and multiple endocrine hyperfunction. An activating missense mutation of the α subunit of the Gs protein (Gsα) was found in several affected tissues, resulting in prolonged stimulation of adenylate cyclase. Our recent study has indicated that the cells derived from the fibrous bone dysplasia tissues in MAS patients produced increased levels of interleukin-6 (IL-6), which may be responsible for the increased bone resorption in this disease. In the present investigation, to analyze the molecular mechanism of the increased IL-6 production by activating mutant Gsα in bone cells, we established mouse osteoblastic MC3T3-E1 cells stably transfected with the activating mutant Gsα expression vector. These cells showed a significant increase of intracellular cAMP levels and produced a higher amount of IL-6 than the cells transfected with control vector or wild-type Gsα expression vector. Analysis of the IL-6 promoter revealed that any of the AP-1, nuclear factor (NF)-IL6, and NF-κB binding elements are important for the activating mutant Gsα-induced gene expression. Electrophoretic mobility-shift assays using nuclear extracts of the mutant Gsα-expressing cells showed that phospho(Ser^{133})-cAMP-responsive element binding protein (CREB), AP-1, NF-IL6, and NF-κB were increased, compared with the control cells or the wild-type Gsα-expressing cells. These results indicate that activating mutant Gsα increases the transcriptional factors binding to CRE, AP-1, NF-IL6, and NF-κB elements to induce IL-6 gene expression in the osteoblastic cells. (J Bone Miner Res 1998;13:1084–1091)

INTRODUCTION

The McCune–Albright syndrome (MAS) is characterized by localized bone lesions termed polyostotic fibrous dysplasia, café-au-lait pigmentation of the skin, and autonomous hyperfunction of multiple endocrine organs.[1–3] Recent observations revealed that an activating missense mutation in the gene for the α subunit of the stimulatory guanine nucleotide binding protein Gs (Gsα) was found in several affected tissues.[4–6] Substitution of Arg^{201} of Gsα protein with either Cys or His causes the inhibition of the intrinsic guanosine triphosphatase activity, resulting in prolonged stimulation of adenylate cyclase. In endocrine organs, this leads to increased production of cAMP and thereby activation of the Gsα protein-associated hormone actions.[7]

In fibrous dysplasia of bone in MAS patients, the activating mutation of Gsα protein has also been found.[6] We have recently shown that there were an increased number of osteoclasts in bone biopsy specimens from MAS patients.[8] In addition, the cultured fibrous cells derived from these patients have been found to produce increased levels of interleukin-6 (IL-6) as well as intracellular cAMP.[8] Since IL-6 is a key cytokine important for the differentia-

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tion of osteoclasts,[9,10] this cytokine is suggested to have a pathogenetic role in the bone lesions of MAS patients. In our experiments, the increased production of IL-6 was inhibited by the specific inhibitor of cAMP-dependent protein kinase, Rp-8-bromo-adenosine 3′, 5′-cAMP (Rp-8Br-cAMP).[11] It suggests that increase of intracellular cAMP levels has a central role for the increased IL-6 production in the bone cells from the patients. However, the signal transduction pathways mediating the IL-6 induction in the bone cells remain unidentified.

In the present study, we have transfected activating mutant of Gsα cDNA into mouse osteoblastic MC3T3-E1 cells. Analysis of the stable transfectants demonstrates that the expression of the activating mutant of Gsα leads to the overproduction of intracellular cAMP as well as the increased secretion of IL-6. These stable cell lines display the activation of multiple nuclear transcription factors, which are found to be critical for IL-6 gene expression.

MATERIALS AND METHODS

Reagents

Alpha minimum essential medium (α-MEM), geneticin (G418 sulfate), and Lipofectamine Plus were obtained from GIBCO Laboratories (Grand Island, NY, U.S.A.). Isobutylmethylxanthine (IBMX) and dibutyryl cAMP were obtained from Sigma (St. Louis, MO, U.S.A.). Rp-8Br-cAMP was purchased from Biology Life Science Institute (Bremen, Germany). rhPTH(1–34) was obtained from Peptide Institute (Osaka, Japan). Rabbit polyclonal antibodies against c-Jun, pan-Jun, c-Fos, nuclear factor (NF)-IL6, NF-κB p65, and NF-κB p50 used in electrophoretic mobility gel shift assay (EMSA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Rabbit polyclonal anti-CREB and antiphospho(Ser^{133})-CREB antibodies were obtained from New England Biolabs (Beverly, MA, U.S.A.). All other reagents were of analytical grade.

Cell culture

Mouse calvaria-derived osteoblastic cell lines, MC3T3-E1,[12] were obtained from the RIKEN Cell Bank (Tsukuba, Japan). These cells were grown in α-MEM supplemented with 10% fetal calf serum (FCS; Bioserum, Victoria, Australia) in a humidified atmosphere of 5% CO_2/95% air at 37°C.

Expression vectors

pSV2-Gsα-WT consists of the wild-type Chinese hamster Gsα cDNA and pSV2-dhfr expression vector. pSV2-Gsα-Q227L contains the constitutively activating mutated ($Glu^{227}\rightarrow$ Leu) Chinese hamster Gsα cDNA. These expression vectors were kindly provided by Dr. J.-P. Loeffler.[13] Gsα-WT and Gsα-Q227L were subcloned into the eukaryotic expression vector pRc/RSV (Invitrogen, San Diego, CA, U.S.A.) to give pRc/RSV-Gsα-WT and pRc/RSV-Gsα-Q227L, respectively. $Arg^{201}\rightarrow$His mutation was introduced into pRc/RSV-Gsα-WT by oligonucleotide-directed site-specific mutagenesis[14] to generate pRc/RSV-Gsα-R201H. The mutation was confirmed by direct sequencing.

Establishment of stable cell lines

MC3T3-E1 cells were plated onto 10-cm culture dishes (5×10^5 cells/dish) in α-MEM containing 10% FCS. At subconfluency, the cells were transfected with 4 μg of the appropriate pRc/RSV-Gsα expression vector or control empty vector (pRc/RSV), using Lipofectamine Plus reagent (GIBCO Laboratory, Rockville, MD, U.S.A.). After 48 h in culture, stable transfectants were selected in 400 μg/ml G418 for 3 weeks. The G418-resistant clones were pooled for further studies.

cAMP measurement

The stably transfected cells were plated onto 12-well plates (2×10^5 cells/well) in α-MEM supplemented with 10% FCS for 24 h before testing. The cells were incubated with 1 mM IBMX at 37°C for 1 h without serum. In some experiments, after the incubation with IBMX for 1 h, the cells were treated with 10 μM rhPTH(1–34) for 15 minutes. After the medium was aspirated, the plates were frozen on dry ice and cAMP was extracted by 0.1 N HCl. The cAMP contents were determined by the enzyme immunoassay system (Amersham Int., Buckinghamshire, U.K.).

Quantitation of secreted IL-6

MC3T3-E1 cells or the stably transfected cells were plated onto 24-well plates (5×10^4 cells/well) in α-MEM containing 10% FCS and cultured to subconfluency. Then the cells were cultured in 0.3 ml serum-free α-MEM for 48 h. In some experiments, the cells were cultured in the presence of 1 mM dibutyryl cAMP or 10 μM rhPTH(1–34). Conditioned media were collected and centrifuged at 3000 rpm for 5 minutes to remove any particulate material. Mouse IL-6 concentrations were measured by specific enzyme-linked immunosolvent assays from R&D Systems (Minneapolis, MN, U.S.A.).

Reporter constructs and luciferase assay

−840IL6LUC is a reporter plasmid consisting of 5′ flanking region (−840/+12) of the human IL-6 gene, directing expression of the reporter gene, firefly luciferase. This reporter plasmid contains AP-1, CREB, NF-IL6, and NF-κB binding sites in the human IL-6 gene promoter. –179IL6LUC contains CREB, NF-IL6, and NF-κB sites but not AP-1 site in 5′ flanking region (−179/+12) of the human IL-6 gene.[15] Three kinds of mutant where the NF-κB site, the NF-IL6 site, or the both sites were disrupted were constructed; the NF-κB site was disrupted by converting GGGATTTTCC to AATATTTTCC, and the NF-IL6 site was disrupted by converting ACATTGCACAATCT to ACACTACAAACTCT.[16]

The stably transfected MC3T3-E1 cells were plated onto 6-well plates (5×10^5 cells/well) in α-MEM containing 10% FCS. Twenty-four hours later, the cells were exposed to 0.5

μg of reporter plasmid, 1.0 μg of seapansy luciferase control plasmid (Toyo Beanet, Tokyo, Japan), and 10 μl of Lipofectamine Plus reagent in α-MEM without FCS for 3 h. Three hours after the addition of FCS to a final concentration of 10%, the medium was replaced with α-MEM containing 10% FCS. Thereafter, the cells were allowed for 48 h. Cell extracts were prepared in lysis buffer and assayed for each luciferase activity in Lumat LB9501 luminometer (Berthold Systems, Aliquippa, PA, U.S.A.).

Nuclear extraction and EMSA

The stably transfected cells were grown in 10-cm culture dishes until they reached 70–80% confluency. The cells were serum-deprived for 24 h, and the nuclear extracts were prepared by the methods of Schreiber et al.[17] EMSA was performed using a gel shift assay kit from Stratagene (La Jolla, CA, U.S.A.). The double-stranded oligonucleotides used as probes are shown below:

CREB:
GATTGGCTGACGTCAGAGAGCT
CTAACCGACTGCAGTCTCTCGA
AP-1:
CGCTTGATGACTCAGCCGGAA
GCGAACTACTGAGTCGGCCTT
NF-IL6:
TCGACACATTGCACAATCTTAAC
GTGTAACGTGTTAGAATTGAGCT
NF-κB:
GATCGAGGGGACTTTCCCTAGC
CTAGCTCCCCTGAAAGGGATCG

Five micrograms of nuclear proteins were incubated with 500 pg of ^{32}P-labeled oligonucleotides for 30 minutes at room temperature. The samples were loaded onto 5% nondenaturing acrylamide gels, according to the manufacturer's protocol. In some experiments, nuclear extracts were incubated with either unlabeled oligonucleotides or corresponding antibodies before the incubation with ^{32}P-labeled oligonucleotides. After electrophoresis, gels were exposed to X-ray films (X-Omat, Kodak, Rochester, NY, U.S.A.).

RESULTS

Gsα stimulation increases IL-6 production in MC3T3-E1 cells

We first examined whether the transient stimulation of intracellular cAMP levels induces the production of IL-6 in mouse osteoblastic MC3T3-E1 cells. In our experimental condition, IL-6 concentration was 14.3 ± 5.6 pg/ml in the culture supernatants of MC3T3-E1 cells. The membrane-permeable cAMP analog dibutyryl cAMP significantly stimulated the IL-6 secretion in these cells (Fig. 1). The increase of IL-6 secretion by dibutyryl cAMP was inhibited by the specific inhibitor of cAMP-dependent protein kinase A (PKA), Rp-8Br-cAMP (Fig. 1). These results indicate that the transient stimulation of cAMP action induces IL-6 secretion by MC3T3-E1 cells in which PKA is involved.

As we reported earlier,[8] in cells isolated from the fibrous bone dysplasia tissues in MAS patients, an activating Gsα mutation (Arg^{201}→His) was identified. These cells demonstrated significant increase of IL-6 production. Thus, we examined the ability of constitutively active mutant Gsα protein to induce IL-6 in MC3T3-E1 cells. For this purpose, we transfected the cells with a wild-type or two kinds of the activating mutant Gsα expression vector. In this study, we used the wild-type, the mutant (Arg^{201}→His), and the mutant (Glu^{227}→Leu) Gsα cDNA expression vectors. The G418-resistant colonies were clonally selected to yield the stable transfectants. Control cell line was obtained by transfection with the control empty vector pRc/RSV (mock-transfectant). The intracellular cAMP content of the control cells was typically 677 ± 90 fmol/well. It was increased to 1.3-fold in stable transfectants with wild-type Gsα and to 4.5-fold in the mutant (Arg^{201}→His) Gsα-expressing cells, whereas it was the highest to 18-fold in mutant (Glu^{227}→Leu) Gsα-expressing cells (Fig. 2A). Addition of 10 μM rhPTH(1–34) increased the intracellular cAMP content to 27-fold in the control cells. The rhPTH(1–34)-dependent induction of cAMP was 12-fold in the wild-type Gsα-expressing cells and 1.8-fold in the mutant (Arg^{201}→His) Gsα-expressing cells, while it was blunted to 0.8-fold in the mutant (Glu^{227}→Leu) Gsα-expressing cells (Fig. 2B).

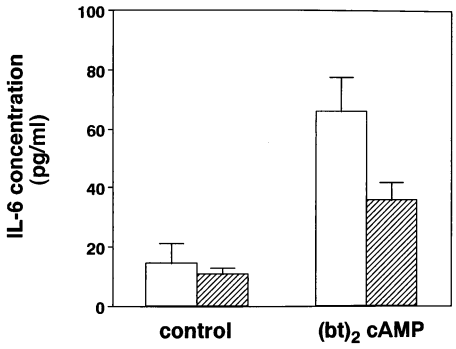

FIG. 1. Effects of transient stimulation of intracellular cAMP levels on IL-6 secretion by MC3T3-E1 cells. The MC3T3-E1 cells were cultured with or without 1 mM dibutyryl cAMP ((bt)2 cAMP) in the presence (shaded bar) or absence (open bar) of 100 μM Rp-8Br-cAMP for 48 h. After the incubation, IL-6 concentrations in the culture supernatants were determined. Bars represent means ± SD in triplicate assay.

Next, IL-6 concentration in the culture supernatants of the transfectants was determined. The mock transfectants (control cells) produced low level (13.9 ± 6.5 pg/ml) of IL-6. The wild-type Gsα-expressing cells displayed a slightly increased amount of IL-6 production. The mutant (Arg^{201}→His) and the mutant (Glu^{227}→Leu) Gsα-expressing cells produced higher amount of IL-6 (153 ± 23.8 and 460 ± 66.8 pg/ml, respectively) (Fig. 3).

Thus, the mutant (Glu^{227}→Leu) Gsα-expressing MC3T3-E1 cells contained a higher amount of intracellular cAMP and produced a higher amount of IL-6 than the mutant (Arg^{201}→His) Gsα-expressing cells. In the following experiments, therefore, we used the mutant (Glu^{227}→Leu) Gsα-expressing cells in order to analyze the molecular

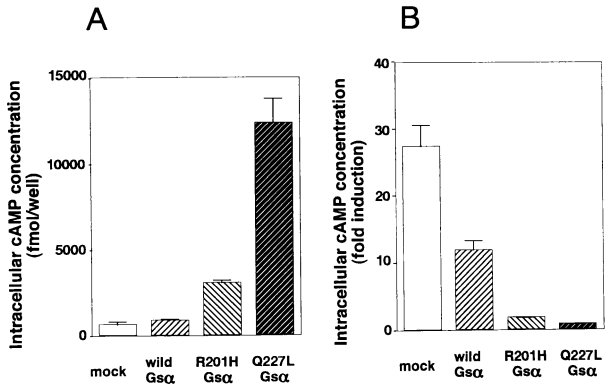

FIG. 2. (A) Intracellular cAMP concentration in MC3T3-E1 cells transfected with Gsα protein-expression vectors. The MC3T3-E1 cells were transfected with empty vector (mock), wild Gsα expression vector (wild), mutant (Arg^{201}→His) Gsα expression vector (R201H), or mutant (Glu^{227}→Leu) Gsα expression vector (Q227L). cAMP concentrations in cell lysates were determined. Bars represent means ± SD in triplicate assay. (B) Effects of rhPTH(1–34) on intracellular cAMP concentrations in MC3T3-E1 cells transfected with the Gsα protein-expression vectors. After the cells were stimulated for 15 minutes with 10 μM hPTH(1–34), intracellular cAMP concentrations were determined. Fold induction was shown as means ± SD in triplicate assay.

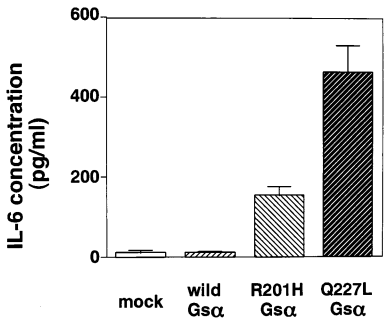

FIG. 3. IL-6 secretion by MC3T3-E1 cells stably transfected with Gsα protein-expression vectors. The cells were transfected with empty vector (mock), wild Gsα expression vector (wild), mutant (Arg^{201}→His) Gsα expression vector (R201H), or mutant (Glu^{227}→Leu) Gsα expression vector (Q227L). IL-6 concentrations in the culture supernatants were determined. Bars represent means ± SD in triplicate assay.

mechanism of IL-6 production enhanced by the mutant Gsα expression.

Factor-binding sites of the IL-6 promoter

Multiple nuclear transcription factor-binding sites of the IL-6 promoter is important to confer transcriptional activation by Gsα stimulation. IL-6 promoter region contains AP-1, CREB, NF-IL6, and NF-κB binding sites.[18] CRE is present very close to NF-IL6 binding elements.[19] To elucidate which *cis*-acting binding elements are important for the activating Gsα mutant-induced IL-6 gene expression, we transfected various kinds of reporter constructs expressing firefly luciferase under the control of the human IL-6 gene promoter into the mutant Gsα-expressing MC3T3-E1 cells. Luciferase activity was determined in cell extracts of these cells. As shown in Fig. 4, –840IL6LUC had the strongest promoter activity in these cells. Deletion below –179 bp caused reduction of the luciferase activity, indicating that the AP-1 site is required for transcriptional activation of IL-6 gene by the mutant Gsα expression. When either the NF-IL6 binding site or the NF-κB binding site was disrupted, the luciferase activity was reduced. When both sites were disrupted, the luciferase activity was dramatically reduced.

Gsα stimulation activates multiple transcriptional factors in MC3T3-E1 cells

To verify the nuclear transcription factors activated by Gsα stimulation in MC3T3-E1 cells, we performed EMSA using the nuclear extracts from the stable transfectants. EMSA of an oligonucleotide probe encoding CRE revealed that the DNA–protein complex was obtained in nuclear proteins of the control cells (Fig. 5A, lane 1). Intensity of the gel-retarted bands was almost unchanged in the wild-type Gsα-expressing cells (Fig. 5A, lane 2). It was slightly increased in the mutant Gsα-expressing cells (Fig. 5A, lane 3). The gel-retarded bands were abolished by excess unlabeled oligonucleotides but not unrelated oligonucleotides (Fig. 5A, lanes 4 and 5). In addition, they were supershifted by addition of anti-CREB antibody as well as antiphospho(Ser^{133})-CREB antibody (Fig. 5A, lanes 6–11). The supershift by antiphospho (Ser^{133})-CREB antibody was preferential in the mutant Gsα-expressing cells (Fig. 5A, lanes 9–11).

When a double-stranded oligonucleotide probe encoding an AP-1 site was interacted with nuclear proteins of the control cells, an apparent DNA–protein complex was detected (Fig. 5B, lane 1). In EMSA using nuclear proteins of the wild-type Gsα-expressing cells, intensity of the migrating complex was barely altered (Fig. 5B, lane 2). By contrast, EMSA using nuclear proteins of the mutant Gsα-expressing cells revealed that the intensity was significantly increased (Fig. 5B, lane 3). The DNA–protein complex band disappeared in the presence of excess unlabeled oligonucleotides but not unrelated oligonucleotides (Fig. 5B, lanes 4 and 5). The band was partially supershifted by addition of anti–c-Jun antibody or anti–c-Fos antibody (Fig. 5B, lanes 6 and 7); the supershift by anti–c-Jun antibody was very faint. However, the band was almost completely supershifted by addition of both anti–c-Jun and anti–c-Fos antibodies (Fig. 5B, lane 8). These were almost the same when anti–pan-Jun antibody was used instead of anti–c-Jun antibody (data not shown).

Analysis of the interaction of nuclear proteins with an oligonucleotide probe containing the consensus sequence for NF-IL6 binding revealed that gel-retarded bands were found in the nuclear extracts from the control cells (Fig. 5C, lane 1). Intensity of the gel-retarded bands was almost

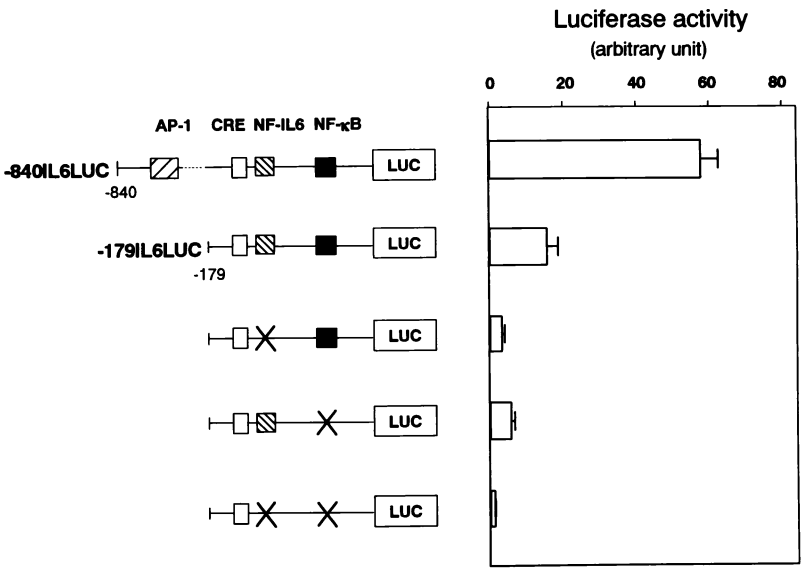

FIG. 4. Characterization of the human IL-6 promoter region involved in mutant (Glu^{227}→Leu) Gsα-induced gene expression. Reporter constructs (0.5 μg) containing various fragments of the IL-6 regulatory region that direct expression of the firefly luciferase gene were introduced transiently to the mutant (Glu^{227}→Leu) Gsα-expressing MC3T3-E1 cells, together with 1.0 μg seapansy luciferase control plasmid. Firefly luciferase relative light units were adjusted to seapansy luciferase relative light units to correct for variations in transfection efficiency. Bars represent means ± SD in triplicate assay.

unchanged in the nuclear extracts from the wild Gsα-expressing cells, whereas it was strongest in those from the mutant Gsα-expressing cells (Fig. 5C, lanes 2 and 3). The specificity of the gel-retarded bands was again confirmed by competition with excess oligonucleotides and supershift with anti–NF-IL6 antibody (Fig. 5C, lanes 4–6). Nonspecific antibody failed to shift the gel-retarded bands (data not shown).

Finally, EMSA using a probe encoding NF-κB binding site demonstrated that specific DNA–protein complexes were obtained in the control cells. Intensity of the gel-retarded bands was higher in the wild-type Gsα-expressing cells and the highest in the mutant Gsα-expressing cells (Fig. 5D, lanes 1–3). The gel-retarded bands disappeared in the presence of excess unlabeled oligonucleotides but not unrelated oligonucleotides (Fig. 5D, lanes 4 and 5). They were partially supershifted by addition of anti-p65 antibody or anti-p50 antibody, and were completely supershifted by the both antibodies (Fig. 5D, lanes 6–8). Nonspecific antibody failed to shift the gel-retarded bands in each experiment of EMSA (data not shown).

DISCUSSION

In this study, we established mouse osteoblastic MC3T3-E1 cells stably expressing activating mutant as well as wild-type Gsα protein. Both the mutant (Arg^{201}→His) and (Glu^{227}→Leu) Gsα-expressing cells had significantly higher levels of cAMP than the control cells (mock transfectants) or the wild-type Gsα-expressing cells (Fig. 2A). In addition, these cells produced significantly higher amounts of IL-6 (Fig. 3). Such characteristics resemble those observed in cells derived from fibrous bone dysplasia tissues in MAS patients.[8] Therefore, the expression of the mutant Gsα protein is suggested to be responsible for the increased cAMP production as well as IL-6 secretion by the fibrous bone dysplasia cells, and the stable transfectant cell lines are suitable models for analyzing the molecular characteristics of these cells. In our study, the mutant Gsα-expressing cells displayed blunted increase of cAMP in response to rhPTH(1–34) (Fig. 2B). Such a characteristic is also similar to that of the cells derived from a MAS patient.[8] PTH is known to activate both the adenyl cyclase signal transduction pathway as well as the phospholipase C pathway.[20] In addition, it has been shown that adenyl cyclase and IL-6 are involved in PTH-stimulated bone resorption.[21] Our study indicates that the constitutive activation of Gsα renders the osteoblastic cells less sensitive to PTH with regards to cAMP induction. Although the molecular mechanisms for such a phenomenon remain unknown, the mutant Gsα-induced continuous stimulation of intracellular cAMP levels may modulate the function of PTH receptor and/or its coupled G-proteins.

IL-6 production is positively or negatively regulated in various cell types by a variety of signals.[18] In fibroblasts and certain tumor cell lines, a variety of cytokines, including IL-1, tumor necrosis factor (TNF), platelet-derived growth factor, and interferon-β enhance IL-6 production. Diacylglycerol- and cAMP-activated pathways also enhance the IL-6 gene expression in fibroblasts.[22,23] Within the 5′-flanking regions of IL-6 gene, sequences similar to transcriptional enhancer elements such as the c-*fos* serum responsive element and the consensus sequences for CRE, AP-1, NF-κB, NF-IL6, and glucocorticoid receptor binding elements are identified.[18,24] Therefore, some nuclear transcription factors binding to such regulatory elements in the IL-6 gene promoter are indicated to play pivotal roles in the increased IL-6 production in specified conditions. In the present study, we tried to elucidate which *cis*-acting regulatory elements are responsible for the increased IL-6 production exerted by activating mutant Gsα. We demonstrated that −840IL6LUC has the stronger promoter activity than −179IL6LUC in the mutant Gsα-expressing cells. Since an AP-1 site is present within the deletion part, an AP-1 site is suggested to be important for the transcrip-

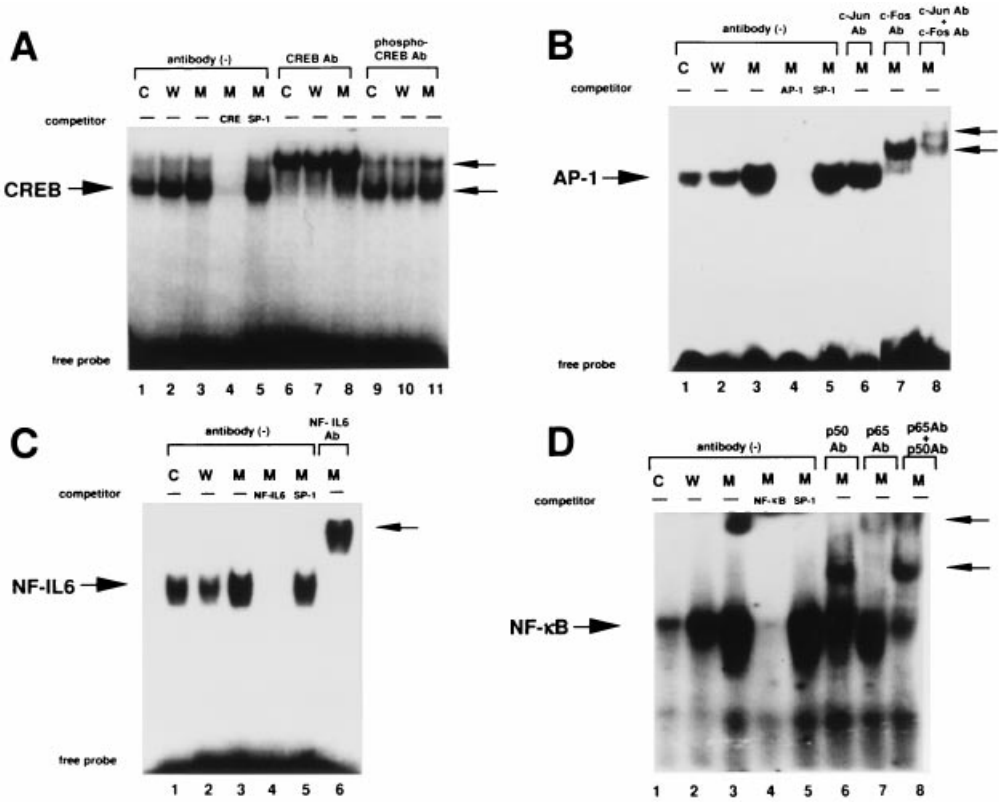

FIG. 5. EMSA for (A) CREB, (B) AP-1, (C) NF-IL6, and (D) NF-κB in the nuclear extracts from MC3T3-E1 cells transfected with Gsα protein-expression vectors. Nuclear proteins were extracted from mock transfectants (C), wild Gsα-transfectants (W), or mutant (Glu^{227}→Leu) Gsα-transfectants (M). (A) EMSA using the oligonucleotide probe specific for CRE binding was performed on the nuclear proteins preincubated with no reagents (lanes 1–3), 50-fold excess of unlabeled CREB probe (lane 4), 50-fold excess of unrelated oligonucleotides (lane 5), anti-CREB antibody (lanes 6–8), or antiphospho-CREB antibody (lanes 9–11). (B) EMSA using the oligonucleotide probe specific for AP-1 binding was performed on the nuclear proteins preincubated with no reagents (lanes 1–3), 50-fold excess of unlabeled AP-1 probe (lane 4), 50-fold excess of unrelated oligonucleotides (lane 5), anti–c-Jun antibody (lane 6), antic-Fos antibody (lane 7), and both anti-c-Jun and anti-c-Fos antibodies (lane 8). (C) EMSA using the oligonucleotide probe specific for NF-IL6 binding was performed on the nuclear proteins preincubated with no reagents (lanes 1–3), 50-fold excess of unlabeled NF-IL6 probe (lane 4), 50-fold excess of unrelated oligonucleotides (lane 5), or anti–NF-IL6 antibody (lane 6). (D) EMSA using the oligonucleotide probe specific for NF-κB binding was performed on the nuclear proteins preincubated with no reagents (lanes 1–3), 50-fold excess of unlabeled NF-κB probe (lane 4), 50-fold excess of unrelated oligonucleotides (lane 5), anti-p50 antibody (lane 6), anti-p65 antibody (lane 7), or both anti-p50 and anti-p65 antibodies (lane 8). At least three experiments of separate EMSA analysis gave the similar results.

tional activation, Furthermore, the disruption of an NF-IL6 site, an NF-κB site, or both sites reduced the promoter activity, indicating that each site mediates the mutant Gsα-induced IL-6 gene expression.

EMSA using specific oligonucleotide probes demonstrated that phospho(Ser^{133})-CREB was increased in the mutant Gsα-expressing cells. NF-IL6, AP-1, and NF-κB were also activated in the nuclear extracts from the mutant Gsα-expressing cells. Thus, continuous stimulation of cAMP formation resulting from the mutant Gsα expression may lead to the activation of the multiple transcription factors in the osteoblastic cells, thereby cause the long-term genomic effects on IL-6 gene expression.

CREB was first identified as a nuclear CRE-binding protein.[25] CREB enhances the transcription of CRE-driven reporter gene by phosphorylation in response to cAMP stimulation, with no apparent change in DNA binding activity.[26] In EMSA, binding to CRE probe was only modestly increased in mutant Gsα-expressing MC3T3-E1 cells. However, the supershift by antiphospho(Ser^{133})-CREB antibody was dominant in the mutant Gsα-expressing cells, compared with the control cells or the wild-type Gsα-expressing cells (Fig. 5A). It indicates that the mutant Gsα induced phosphorylation of CREB at Ser^{133}, causing an increase in transactivation. In this context, an oncogenic mutant Gsα and overexpressed wild Gsα were found to induce the Ser^{133}-phophorylation of CREB and cAMP-responsive gene expression in human somatotroph adenomas.[27]

AP-1 consists of homodimers of Jun family proteins (c-

Jun, JunB, and JunD) or heterodimers of the Jun family with Fos family proteins (c-Fos, FosB, Fra-1, and Fra-2).[28] It has been shown that PKA increased AP-1 binding in a cell type–specific manner; the effect was observed in NIH-3T3, COS, and JEG-3 cells but not in HeLa or HepG2 cells.[29] In our experiments, activating mutant Gsα expression led to an increase of AP-1 binding in MC3T3-E1 cell nuclear extracts (Fig. 5B). The shift with anti–c-Jun or anti–pan-Jun antibody was very faint. However, the inclusion of anti–c-Fos antibody and anti–c-Jun antibody almost completely shifted the AP-1–DNA complex. Thus, the activated AP-1 complex in the mutant Gsα-expressing MC3T3-E1 cells are suggested to be composed at least of c-Jun and c-Fos, although other components such as the other Jun family or ATF family members may participate. In this relation, Candeliere et al.[30] clearly demonstrated that increased c-*fos* expression was detected in the bone lesions from the patients with fibrous dysplasia. The increased AP-1 activity by cAMP-dependent signal transduction pathway might be resulted from the transcriptional induction of *jun* and *fos* gene families[24,31–33] and/or post-translational modifications.[34,35]

NF-IL6, also called as C/EBPβ, is thought to be involved in induction of several cytokine genes, such as IL-6, IL-8, and TNF-α.[17] In this study, the activating mutant Gsα-expression in MC3T3-E1 cells resulted in an increase of NF-IL6 binding to its recognition elements (Fig. 5C). PKA has been shown to cause increased phosphorylation and nuclear translocation of NF-IL6 in rat pheochromocytoma PC12 cells.[36] By contrast, PKA induced in vitro phosphorylation of NF-IL6 in the region between Ser^{173} and Ser^{223} and at Ser^{240} reduces its DNA binding, while phosphorylation of Ser^{105} has no effect on DNA binding.[37] Thus, the molecular mechanism in which the mutant Gsα-expressing MC3T3-E1 cells elicit NF-IL6 activation still remains unknown. PKA-induced phosphorylation in vivo may provide a different mechanism for the activation of NF-IL6.

NF-κB is a heterodimer consisting of two proteins encoded by the *rel* gene family.[38] NF-κB was found to be important for the transcriptional regulation of the IL-6 gene,[39,40] and in vivo targeting of the p50 (NFKB1) subunit resulted in reduced expression of the IL-6 gene.[41] In our experiments, NF-κB consisting of p65 and p50 subunits were found to be significantly activated in the mutant Gsα-expressing MC3T3-E1 cells. Recent observation suggested that PKA-mediated phosphorylation of NF-κB is involved in the activation of NF-κB.[42] In this context, the transcriptional activity of NF-κB was found to be regulated by an IκB-associated PKA catalytic subunit through a cAMP-independent mechanism.[43]

MC3T3-E1 cells stably expressing activating mutant Gsα provided us the information that mutant Gsα expression per se is enough to induce IL-6 production in the osteoblastic cells. In addition, these cells showed increased phosphorylation of CREB, and the increase in AP-1, NF-IL6, and NF-κB binding to their recognition sequences. Thus, activating mutation of Gsα protein may lead to increased IL-6 production via the activation of the multiple transcription factors in fibrous bone dysplasia tissues in MAS patients.

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